

Genetic variation of xylem formation in norway spruce (*Picea abies* (L.) Karst.) clones with contrasting growth rhythm.

Genetisk variasjon i veddannelse hos grankloner (*Picea abies* (L.) Karst.) med kontrasterende vekstrytme

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Preface

With this, I finish my master's degree in forestry at the Norwegian University of Life Sciences (UMB), at the Department of Ecology and Natural Resource Management (INA). It is the final task after five years of university education. This has been an interesting topic to work with and I got the opportunity to learn more about genetics and wood formation in Norway spruce (*Picea abies* (L.) Karst.). My fieldwork led to great days out in the field, and I even got to go to Finland to get some experience in laboratory- and fieldwork from the researches at Metla.

The thesis is a smaller part of a project called WOVEN, «Wood formation under varying environmental conditions». Three countries are representing the project; Norway, Sweden and Finland, the latter which is represented by Harri Mäkinen and the Finnish Forest Research Institute (Metla) is the founder and the main manager of the project. The countries also contain three academic partners and two research institutes. The Norwegian University of Life Sciences (Department of INA) is one of the academics and the Norwegian Forest and Landscape Institute (NFLI) is one of the research institutes.

I would like to thank my supervisor, Professor Olav Høibø at the Norwegian University of Life Sciences and my support supervisor from The Norwegian Forest and Landscape Institute, Scientist Arne Steffenrem. My supervisors are very knowledgeable and they have given me great inspiration. They have also been very helpful with some of the complicated statistics. Thanks to Tore Skrøppa for establishment of the experiment and for good input in the initial phase. And thanks to Geir Østreng for identifying the experiment. Great thanks to Senior Researcher Harri Mäkinen who has served as our cooperation partner in Finland (Metla). He has contributed with good guidance and help with the research equipment. In this connection I would also like to thank Toumo Kalliokoski and María de las Heras from Metla for having me there and teaching me the process from sampling to paraffin embedding and the following sectioning with microtome.

Also great thanks to Hilde Kolstad and Elin Ørmen for good guidance and help at the microscope lab, and Hanna Høibø who has been helping out with some of the razorblade cutting and counting. Last I want to thank the other students at the reading room and especially Ingvild Torsdal for good discussions and advices during the writing process and to Ludvig Fjeld for good support during the whole project.

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Abstract

Genetic variation in the progress of xylem formation and relationships with bud development in Norway spruce (*Picea abies* (L.) Karst.) were studied during one growing season in a clonal trial in southeastern Norway. Also the initiation of latewood formation, and the consequences for latewood percentage was studied. The study site was a clonal trial established as a classical randomized complete block design eliminating some of the site variations in growth conditions. At year 20 from stand establishment, micro-cores were extracted once a week from 16 trees representing four different clones, with known ranking of apical growth rhythm in the spring (bud flush). The sampling were continuously during the growing season from May until October 2010. Tracheid formation started in the beginning of May and ceased in August. The four clones studied were known from measurements at a very juvenile age to be contrasting in respect to timing of bud flush. One flushed very early, one very late and two were more intermediate. This were confirmed by registrations made now and also at age 20, there were significant differences between them ($p < 0.05$). However, no significant relationships were found between the timing of bud flush and wood formation. The different phases of wood formation were measured with the result of some significant differences ($p < 0.05$) between clones in numbers of formed tracheids in the later phases of growing season. One clone that were flushing late were found to form the highest number of tracheids but at the same time the narrowest annual ring in 2010. There were no significant differences in initiation of latewood formation, neither in latewood percentage between the clones studied ($p > 0.05$). Thus the ones with the narrowest ring width did have greater latewood percentage. The results revealed genetic variation between the clones studied with one particular clone showing significantly higher number but narrower tracheids. Thus genetic variation in the progress of xylem formation was found, but this genetic variation seems to be fairly independent from the genetic variation in bud flush. However, this offers opportunities for further research.

This study also contained a methodological study of techniques for preparation of micro-cores in the laboratory, which resulted in a recommendation of the razorblade cutting method. It was both timesaving and sufficiently accurate.

Sammendrag

Genetisk variasjon i veddannelse forløp, samt sammenhengen mellom knoppskyting og veddannelse, hos gran (*Picea abies* (L.) Karst.) ble studert gjennom én vekstsesong i et klonforsøk på Østlandet. Dato for seinveddannelse og andel seinved i forhold til total årringbredde ble også målt. Forsøksbestandet var etablert som en klassisk komplett blokkdesign med tilfeldig utplassering av materialet, og hvor en del av variasjon i vekstvilkår kunne elimineres i den statistiske analysen. Tjue år etter etableringen av forsøksbestandet, ble små boreprøver samlet inn en gang i uken fra fire forskjellige klon, representert ved 16 trær med kjent vekstrytme i skuddskyting om våren. Prøveinnsamlingen ble utført gjennom hele vekstsesongen fra mai til oktober i 2010. Celledannelsen startet i begynnelsen av mai og opphørte i august. Klonenes vekstrytme i forbindelse med skuddskyting var kontrasterende og kunne deles inn i fire, der en av klonene representerte en henholdsvis tidlig skuddskyting, en annen en forholdsvis sein skuddskyting og to med skuddskytingsfase mellom disse igjen. Dette ble gjort på bakgrunn av vekstrytmeregistreringer i forsøksfelt mens plantene var svært unge. Nå var det fortsatt signifikante forskjeller mellom klonene ($p < 0.05$) i skuddskyting registrert i 2010. Ingen tette sammenhenger ble funnet mellom tidspunkt for skuddskyting og forløpet av veddannelse om våren på klonnivå. De ulike fasene i veddannelsen viste vesentlige forskjeller ($p < 0.05$) mellom klon i antall dannede vedceller i de seinere faser av vekstsesongen. Det viste seg at det klonet som hadde sein skuddskyting hadde smalest årringbredde, men dette kan være kontrollert av helt andre gener enn de som bestemmer tidspunkt for skuddskyting. Det var ingen signifikante forskjeller i start av seinveddannelse, ei heller i andel produsert seinved mellom klonene ($p > 0.05$). Imidlertid viste det seg at de klonene som hadde smalest årringbredde også hadde størst andel seinved. Resultatene viste at det var genetiske variasjoner å finne mellom klon i forbindelse med veddannelsen, spesielt for en av klonene som viste seg å ha betydelig flere men smalere celler enn de andre. De genetiske forskjellene syntes i midlertidig å være relativt uavhengig av den genetiske variasjonen i skuddskytingen. Resultatene åpner muligheter for videre forskning.

Oppgaven inneholdt også en metodisk studie av ulike teknikker for fremstilling av vedprøver i laboratoriet. Dette resulterte i at den såkalte barberblad kutte metoden kan anbefales videre, da dette var både tidsbesparende og tilstrekkelig nøyaktig.

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1. Introduction

1.1 Background

Norway spruce (*Picea abies* (L.) Karst.), together with Scots pine (*Pinus sylvestris*) are the most dominating and economically important tree species in Norwegian and north European forests. The species are important as wood raw material in the industry for lumber, pulp and paper. From the last ten years, the annual harvesting of industrial roundwood for sale in Norway have been between 6.6 – 8.2 mills m³ a year (SSB 2011).

Changes in climate conditions and what it brings, has become more and more of an interest during the last decades. Correct silviculture is important to obtain high production and yield in forestry. Wood quality is important, as well as high volume production and a short rotation age. As the climate is expected to get warmer, the mean annual temperature would increase, leading to longer growth periods. It is therefore beneficial to get a deeper insight into the environmentally and genetically related control of wood formation and the properties of wood (Anonymous 2006). This will help reducing the risk of growing Norway spruce with undesirable properties by choosing appropriate genetic materials, and to better the silvicultural guidelines for producing desired wood properties. See chapter «Tree Breeding» below for more detailed information.

The main goal of the research within the WOVEN project, «Wood formation under varying environmental conditions» is to gain a deeper understanding of which factors that are affecting wood formation, and the variation in wood quality of Norway spruce and Scots pine. They want to analyze the extent to which climate has an effect on the wood formation, fiber properties and wood chemistry. The part in the project this thesis will be focused on, involves analyzing the genetic variation in a breeding population of Norway spruce with the study material of trees from four different clones with contrasting growth rhythm. This was a «man-made» range of individuals with good characteristics for studying growth and adaption to climate changes.

Genetic variation within a population and between populations is for instance studied for the properties density and wood formation as such (E.g (Hannrup et al. 2004; Hysten 1997; Steffenrem 2008) However good dating to when the wood formation occurs during the growing season is incomplete, and we know little about the genetic variation in this. This study is preformed to understand this connection better, including how it all fits together in the spring.

A good way to study secondary growth of living trees is to extract small wood samples, called micro-cores, repeatedly during the growing season (Baucker et al. 1998; Forster et al. 2000; Mäkinen et al. 2003; Rossi et al. 2006).

When it comes to the preparation of samples for measurements, there is more than one method to be used. This study also contains a development of methodology. Three different methods were evaluated and tested out. See section, «Test of Methodology for Sample Preparation» in Materials and Methods. The choice of method was based on time availability and the quality that it gave, satisfying our needs.

1.2 Theory

1.2.1 Wood Formation

Wood formation is a designation of both axial and radial increment during the growth period. Trees need a certain temperature sum to start cell division and the wood formation is controlled by auxin (a type of phytohormone) production in the top crown (Larson 1969). The cambium is a structure, which produces new cells to the phloem and the xylem (Table 1 and Figure 1). The activity of the cambium depends on endogenic and exogenic factors such as genotype, phytohormones, photoperiode, temperature, habitat, climatic variations, silviculture, and interactions between these factors (Plomion et al. 2001; Savidge 1996). Products of the photosynthesis are transported through the phloem and water is absorbed from the ground through the roots and the xylem (Taiz & Zeiger 2006). The trunk's function is to store and transport the water from the roots to the crown, and to take the products from the photosynthesis, the nutrients, from the crown and back to the trunk and the roots (Bowyer et al. 2003).

The growth ring consists of xylem cells, which are formed in the cambium. The formation can be divided in three stages, cell division, cell stretching and maturation (Schweingrüber 1988).

Differentiating xylem cells are formed from dividing xylem mother cells and further continuously enlarging and maturing. When the cell has got its final size, the secondary cell wall is formed and lignified. Optimal temperature for cell wall stretching and formation in Norway spruce is according to Horacek et al. (1999) respectively 13 °C and 20 °C. The critical temperature limit is 5± °C. Research by Gindl et al. (2000) confirms that warm summers give wider growth rings with increasing latewood density. Temperature and light as well as access to soil water affects the time each xylem cell spends at different stages of growth, which is crucial to both year ring width and density development (Zimmermann et al. 1971).

The main organic compounds in Norway spruce wood are 40 – 44 % cellulose, 20 – 23 % hemicelluloses and 25 – 35 % lignin (Bowyer et al. 2003). The proportion of lignin is highest in the middle lamella and the primary wall, while the proportion of cellulose is greatest in the secondary wall (Kucera 1998).

The year ring is divided into earlywood and latewood (Mork 1928). The earlywood cells have a short lifetime, only a few days and are characterized by a thin cell wall and big cell lumen. Larson (1969) had a theory that formation of earlywood is happening from when the new shoot is actively developing and elongating, and the transition to latewood occurs about the time when the bud elongation have stopped, sets and harden. Latewood cells can live up to 2-3 months after they are formed, and are not dying before the growth period ends. The shape of the latewood cells are flatter and the cell walls thicker than the earlywood cells because of the longer period with secondary growth (Schweingrüber 1988).

In this study we have been focusing on the formation of xylem, not the phloem or cambium as such. When counting cells for analyzing, only the first enlarged cells «xylem mother cells» (Table 1) were included.

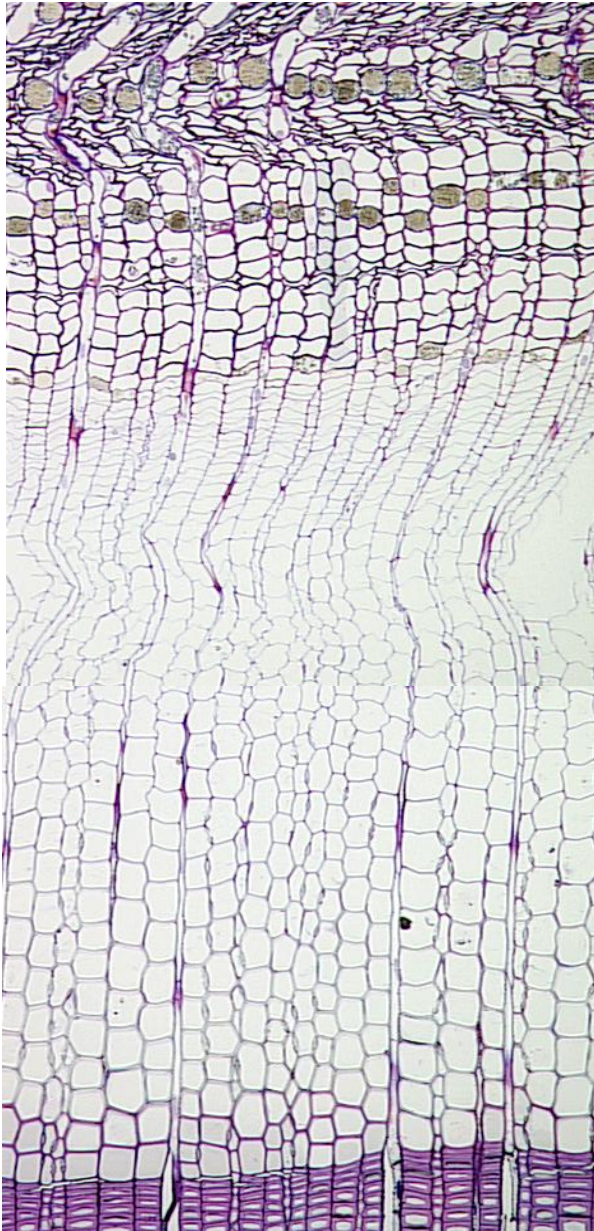


Figure 1. An illustration of cells in different developing stages in the secondary cambium (Table 1). Cross section of the total growth ring, at date 23^d of June. The new growth ring lies between the bark and the xylem woody tissue. At the bottom the clear border shows previous year's growth ring.

Table 1. Secondary cambium. An overview of the division in cambium, in the zone between the bark and the wood. Figured by Zimmermann et al (1971) (after Wilson, *et al.* 1966).

Mature phloem		
Differentiating phloem	Maturing phloem	
	Radially enlarging phloem	
	Dividing phloem (Phloem mother cells)	Cambial zone
Cambium	Cambial initial (dividing)	
Differentiating xylem	Dividing Xylem (Xylem mother cells)	
	Radially enlarging xylem	
	Maturing xylem	
Mature xylem		

1.2.2 Density

Wood density or the weight volume relationship is a key factor when talking about wood quality and it is the most examined wood property. Density is important for both pulp and paper products and different solid wood products. Density varies within the tree depending on age of cambium and position relative to crown, and between trees due to variation in growth conditions, such as soil fertility and competitive and genetics background (Savidge 1996). Silviculture regime is also affecting

the wood density. For instance, a northern provenance moved south is likely to start the growth earlier in the spring than the local ones. This makes them exposed to the frost in the spring, but not in the autumn (summarized in Edvardsen & Steffenrem 2010). It is also found that this transferring makes them form a greater percentage of latewood, thus better density (Kollmann & Côté 2007). Moving from south till north gives opposite outcomes. Southern provenances are prepared for a long autumn but will not have time to finish the cell formation when the growth stops due to the winter.

Since the cell walls have the same density for most species the proportion of cell wall to cell lumen is decisive for the density. In soft wood like Norway spruce therefore the cell wall thickness and the latewood percentage of the annual ring are the most important factors for the wood density. Cross sectional size is also important. In soft wood species density normally decreases with increasing growth ring width (Bowyer et al. 2003). The proportion of latewood decreases too. While the density increases with increasing height above ground (Wilhelmsson et al. 2002). This is especially the case for spruce, but is not the case in pine where the density is the highest at the bottom of the stem. A great amount of precipitation towards the end of the growing season gives increasing latewood, which causes the density increases (Zimmermann et al. 1971).

1.2.3 Plant Breeding and Genetic Variation

Regeneration after final felling is an essential part of the silviculture, both for making appropriate selections of regeneration materials and to avoid economic losses (summarized in Steffenrem & Kvaalen 2010). The tree species selections that are taken are based on site quality and location.

Genetic variation in Norway spruce is affected by immigration history, natural selection, mutations, pollen and seed dispersal, human activity and coincidences (Skogfrøverket 2010a; Zobel & Talbert 2003). The Norwegian seed breeding program, that is managed by the Norwegian Forest Seed Center aim to use the genetic variation of important growth and wood quality traits to produce seeds with superior genetic characteristics for forest re-establishment. The processing work for spruce in Norway started already at the end of the 1950s. The strategic goal in Norwegian plant breeding towards 2040 is to exploit the genetic variations to establish new seed plantations with better properties for mortality, volume production and wood quality for each locality, without deteriorating the future forest genetic variation (Myking & Skrøppa 2001; Skogfrøverket 2010b). The site index will rise with one class as tested seeds from first generation tree breeding have shown to increase the average stand productivity with 10-20 % (Skogfrøverket 2010b). Improved seeds are better adapted to a changing climate and give a larger carbon sequestration (Kvaalen 2010) as well as less root rot, injuries and stem errors, better insect and pathogen resistance and stem form than seeds from

natural forest stands. To gain greatest possible yield from the forest a combination of right silviculture practices, as in number of plants per hectare, and output density is in addition to the processed seed materials important to control and get satisfying quality properties (summarized in Steffenrem & Kvaalen 2010).

The challenge is to establish to what extent the positive properties are coming from the genes and not environmental influences at the growth site (summarized in Tollefsrud et al. 2010). A lot of testing and observations of the offspring are made at different sites over a longer period.

The influence by genetic factors is varying between the properties. Fifteen per cent of the variation in growth is genetically determined, while 85 % is determined by various environmental effects (Tollefsrud et al. 2010). In the short term environmental factors can be affecting the growth individually on tree level (Denne & Dodd 1981; Larson 1994). According to Skrøppa (2003) growth rhythm is genetically determined and governed by climatic factors such as temperature, day length, water supply and light intensity. Together with factors like nutrients and wind, the environmental factors with the contribution of genes determine the tree's overall appearance (phenotype).

Important growth and quality characteristics that are mainly genetically controlled include height growth and growth rhythm and the most genetically controlled property is the start of apical growth in the spring. Latewood density is to a certain extent genetically controlled (Briffa et al. 1998; Zamudio et al. 2005), but the diameter growth shows to be more environmentally influenced than for instance height growth. Density is therefore better optimized by silviculture (Tollefsrud et al. 2010). Further, growth termination and hardiness in the autumn is also genetically determined, but seems to be more affected by environmental conditions than the timing of budflush (start of apical growth).

In natural populations there are continually and systematically variations from south to north and from low to high altitudes in the time of start and cessation of growth (Edvardsen & Steffenrem 2010). Spruce plants from more northern and high altitude provenances flushes earlier in the spring and has a shorter stretch of growth period than plants from more southern provenances. The early flushing brings a risk of frost damage in early summer.

Seeds produced on the same mother tree in a warm climate are giving plants with a more southerly growth rhythm than plants from seed produced in a cooler climate. Basically it seems that it would be optimal to use seeds that have been produced at a site that is warmer than the site where they are to be grown (Edvardsen & Steffenrem 2010).

By using late flushing materials, it appears to provide benefits in terms of better growth and frost damage tolerance to younger plants. It is however important to know whether use of such materials could potentially have an impact on wood quality, and the trees' ability to survive in the long term.

1.3 Purpose of Study

The objective of this study was to assess the progress of xylem formation and find relationship between bud development and wood formation in different Norway spruce clones with contrasting apical growth rhythm in respect to timing of bud flush. Hopefully the results will be helpful for the further selection and utilization of improved materials in the Norwegian forest tree breeding program.

The goal was to study how the apical growth rhythm was related with the growth rhythm in the stem, and if the correlation between the «early» and «late flushing» clones were close. Further, the goal was to test whether early and late bud flush had an impact on the timing of xylem formation. Did early bud flushing also mean an early cambial activity? The purpose was also to find approximate dates for when the cell formation started and ended in the growing season, when the transition between earlywood and latewood occurred, and how this affected the latewood percentage. It was also of interest to study how well the secondary wall formation was completed at the end of the growing season for the so-called «late flushing» clones.

Further, it was also interesting trying to establish a good method for similar future studies.

2. Materials and Methods

The genetic material studied was developed from a mating design study where pollen from ten individuals were crossed with ten unrelated individuals (Table 2). Five of both female and male parents were of Norwegian and Eastern European origin. The east-European parents were selected from the international provenance trial planted in 1942 at Södra Bäcksjö, Sweden (on the basis of growth and quality characteristics (Skrøppa)). The Norwegian parents originated from natural stands in southern Norway. All parents were selected as plus trees in the breeding program on the basis of growth and quality characteristics. Stange seed orchard was established by grafting sections from the plus trees on root stocks. Crosses were performed in the seed orchard and controlled by isolating female flowers with pollen bags, and then injecting pollen from known father several times. This generated 100 full sib families (Table 2). A thorough description of the cuttings and the procedures are found in (Johnsen & Skrøppa 1992). From the entire set of 100 families (Table 2) a clonal testing program was performed on 20 of the families (Johnsen & Skrøppa 1992).

Table 2. The factorial mating design with Norwegian and Eastern-European parents. Clones were propagated by cuttings from 18 seedlings within each of the 20 full-sib families that are indicated in darker colored-cells. Clones selected from this study was propagated from family 9 (clone 337), 48 (clones 320 and 323) and 43 (clone 127).

			Father									
			Norwegian					Eastern-European				
			713	2037	87	39	6264	5444	5448	5468	5453	5466
Mother	Norwegian	1589	6	7	8	9	10	1	2	3	4	5
		1641	16	17	18	19	20	11	12	13	14	15
		1895	26	27	28	29	30	21	22	23	24	25
		2027	36	37	38	39	40	31	32	33	34	35
		2054	46	47	48	49	50	41	42	43	44	45
	Eastern-European	5440	56	57	58	59	60	51	52	53	54	55
		5441	66	67	68	69	70	61	62	63	64	65
		5443	76	77	78	79	80	71	72	73	74	75
		5451	86	87	88	89	90	81	82	83	84	85
		5460	96	97	98	99	100	91	92	93	94	95

The clones studied (Table 3) were chosen from the 20 families on the basis of known contrasting apical growth rhythm in the spring. Two early and two late flushing clones were selected. The early flushing clones are «320» and «337». They were propagated from the full-sib families 48 and 9

(Table 2), respectively. The clones with late bud-flush are «127» and «323». They were propagated from the full-sib families 43 and 48, respectively. Early flushing clone 320 and late flushing clone 323 are hence full-sibs. Clone 127 (from family 43) is half-sib with clone 320 and 323. As showed in Table 3, both the clone 320, 323 and 337 originates from Norwegian parents (N), while clone number 127 is in addition originating from a father of Ukrainian origin (E).

Table 3. Origin and apical growth rhythm of the four Norway spruce clones. Crossings by Norwegian (N) and Eastern-European parents (E).

Clone	Crossing	Mother	Father	Growth rhythm
127	N-E	2054	5468	Very late bud development in the spring
320	N-N	2054	87	Early bud development in the spring
323	N-N	2054	87	Late bud development in the spring
337	N-N	39	1589	Very early bud development in the spring

2.1 Field trial

The field trial (60°19'54"N; 11°03'06"E) where the material was taken from is located in Nannestad municipality, 60 km north of Oslo, southeastern Norway. A fertile forest floor represents the site quality and the original stands surrounding the study area is dominated by Norway spruce. The site index is approximately G23 using the H40-system (Tveite 1977). The mean temperature from May till October 13, the sampling year, was 12.3°C and the July mean 16.8°C. The amount of precipitation was almost 470 mm for the same period, of which 115 mm came during July (Figure 3). The meteorology data were measured 15 km south of Nannestad (Norwegian Meteorological Institute 2010). The trial at the study site is 20 years old, and was planted with Norway spruce in the period; 26-27th of April, 1990. The plant spacing was two by two meters. The experimental design was a «single-tree-plot» consisting of 6 square replicates with one ramet from each clone planted in all replicates. The cutting experiment contained 175 clones of Norway spruce.

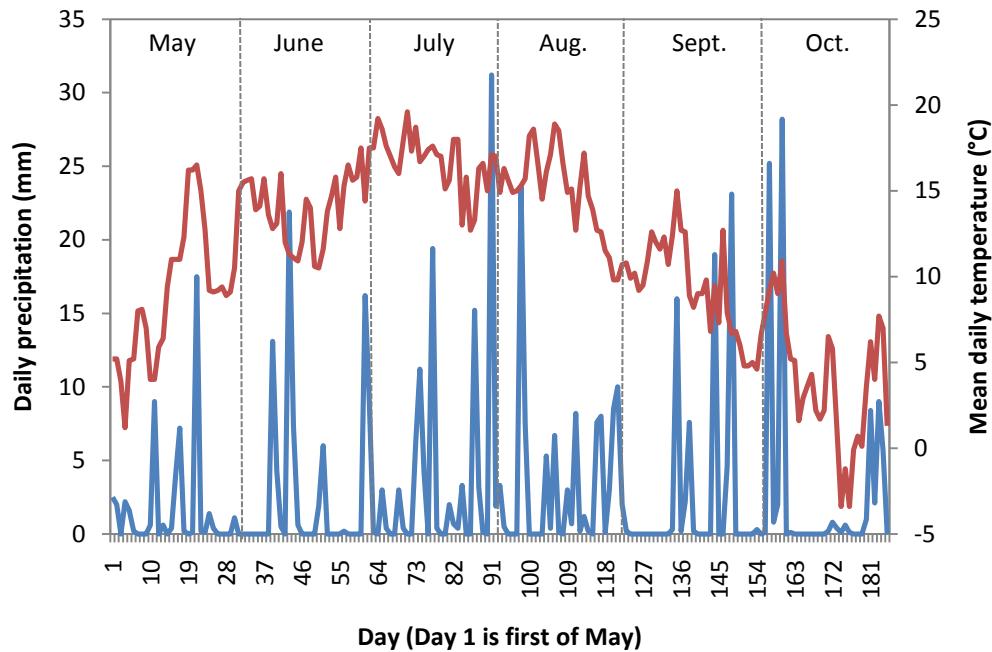


Figure 3. Weather data from the period May till October 2010. Consisting of mean daily temperature ($^{\circ}\text{C}$), red line and daily precipitation (mm), blue line.

2.2 Fieldwork

2.2.1 Selection

Four ramets out of four different clones, from five different replicates were selected. Table 4 shows which trees are selected and their identification number. The mean height of the selected ramets was 12.6 meters, and the mean stem diameter at breast height was approximately 146 millimeters. The replicate number gives an account of where they are located in the field. The selection of which clones to be used was randomly selected, apart from one given condition that was determined. The ramets were chosen randomly, four out of six from one representing clone. If it occurred that one of the ramets was missing because of mortality, we chose to sample from the first next ramet we came by.

Table 4. Characteristics of the sample trees, and which replicate they were placed in. Spot treatment; early start (*) and late start (+).

Replicate	Tree	Clone	Stem diameter at breast height (mm)	Height (m)
1	1	s. 320 *	119	11.8
	2	s. 323 +	139	13.1
	3	s. 127 +	170	13.1
2	4	s. 337 *	158	12.9
	5	s. 320 *	115	12.2
	6	s. 323 +	119	12.2
	7	s. 127 +	145	13.6
3	8	s. 127 +	124	11.7
	9	s. 337 *	150	12.3
	10	s. 323 +	128	11.9
4	11	s. 337 *	168	13.0
	12	s. 320 *	153	12.3
	13	s. 323 +	157	12.5
	14	s. 127 +	164	13.1
5	15	s. 337 *	178	13.1
	16	s. 320 *	153	13.0
Mean			146	12.6

2.2.2 Tree Registrations

Micro-cores, xylem samples were extracted once a week from the 16 spruce trees during the growing season of 2010. First sampling date was 12th of May, just before the trees started flushing and until the end of the growing season. Last sampling date was 13th of October. 608 xylem samples were sampled during this period. By using the Italian tool «Trephor» (Rossi et al. 2006) the xylem samples could be removed without causing any severe physiological impacts to the living tree (Forster et al. 2000). The size of the microcore was 2 mm in diameter and 15 mm in length. A few inches of the outer bark was removed before punching the «Trephor» with a hammer into the stem. The samples were taken at breast height from south, then clockwise all the way around the stem and kept in small individual boxes with ethanol or fix solution for preservation. If the stem was too thin to get all samples in one round, we went 0.3 m down on the stem, with the new starting point from the south direction. To avoid any negative effect caused by previous interventions (Forster et al. 2000), a zigzag pattern and a horizontal distance between the adjacent samples of 25 - 30 mm (Mäkinen et al. 2008) were maintained. Every 4th week samples were taken in all four compass directions, 0.3 m up on the stem from the main sampling area at breast height.

Following tree data were also registered; minimum and maximum diameter at breast height, tree height (measured by a vertex hypsometer) and registration of bud development in the spring and autumn to see if there was a correlation between the bud development and the wood formation. The classification of bud development (Figure 4) in spring was made by using an 8-level scale (Krutzsich 1973) where 0 = dormant buds, 1 = slightly swollen, 2 = buds swollen, 3 = burst of bud scales, 4 = first elongation of needles to about double bud length, 5 = first spread of needles, 6 = elongation of shoot, 7 = differentiation of shoot and 8 = all needles more or less spread. In the autumn we recorded if there were any lamma shoots.



Figure 4. Classification of bud development by using an 8-level scale (Krutzsich 1973). Illustration by Anne Dieset.

2.2.3 Storing and Fixation of Micro-cores

To keep the samples intact for observations, the micro-cores were immediately covered with a fix solution (1.25 % glutaraldehyd, 2 % paraformaldehyd and 0.1 M Pipes-buffer) or ethanol, and stored in a refrigerator at 4°C. (Ethanol is convenient to use if the further sample preparation consists of «paraffin embedding», unless the fix solution is a better solution). After fixation the fix solution was replaced by 0.05 M PIPES-buffer. (When using ethanol, the samples remained in the ethanol through storing).

2.3 Laboratory work

2.3.1 Test of Methodology for Sample Preparation

Three different methods were tested to find the most appropriate way to prepare the samples. An embedding process included either «paraffin embedding» or «plastic embedding» which gave accurate and leveled slices with a thickness on a micrometer level as needed. This methods is however quite comprehensive and time-consuming. The «Razorblade cutting» method was much more time-efficient, and the slices gave satisfactory pictures good enough for our purposes. Precise,

steady and accurate handwork was temporarily decisive because especially the fragile cells in the cambial zone is most vulnerable for cutting (Rossi et al. 2006). Razorblade cutting was as follows our method of decision.

Paraffin Embedding

Paraffin embedding was used in accordance with methods developed by the Metla research group in Finland (Jyske Unpublished). They used paraffin as embedding to avoid damage in the micro-core when slicing with microtome. From the field the micro-cores were closed into bio-cassettes and then dehydrated in a graded series of ethanol from 70 % till 100 %. After dehydration they were cleared with Tissue-clear (xylene substitute) and embedded in paraffin at 65°C. The blocks were trimmed, immersed in water to lubricate the woody tissue and then cut using a rotating microtome. The samples were further cleared of paraffin with Tissue-clear and ethanol and stained with the colors Astra- or Alcian Blue and Safranin (Gerlach 1977). For the measurements of cell formation and differentiation they used a conventional light-microscope connected to a video camera. UV-light was also used for determining the cells, which had started lignification. The preparation with this method was very comprehensive, and time consuming, especially if the person was inexperienced.

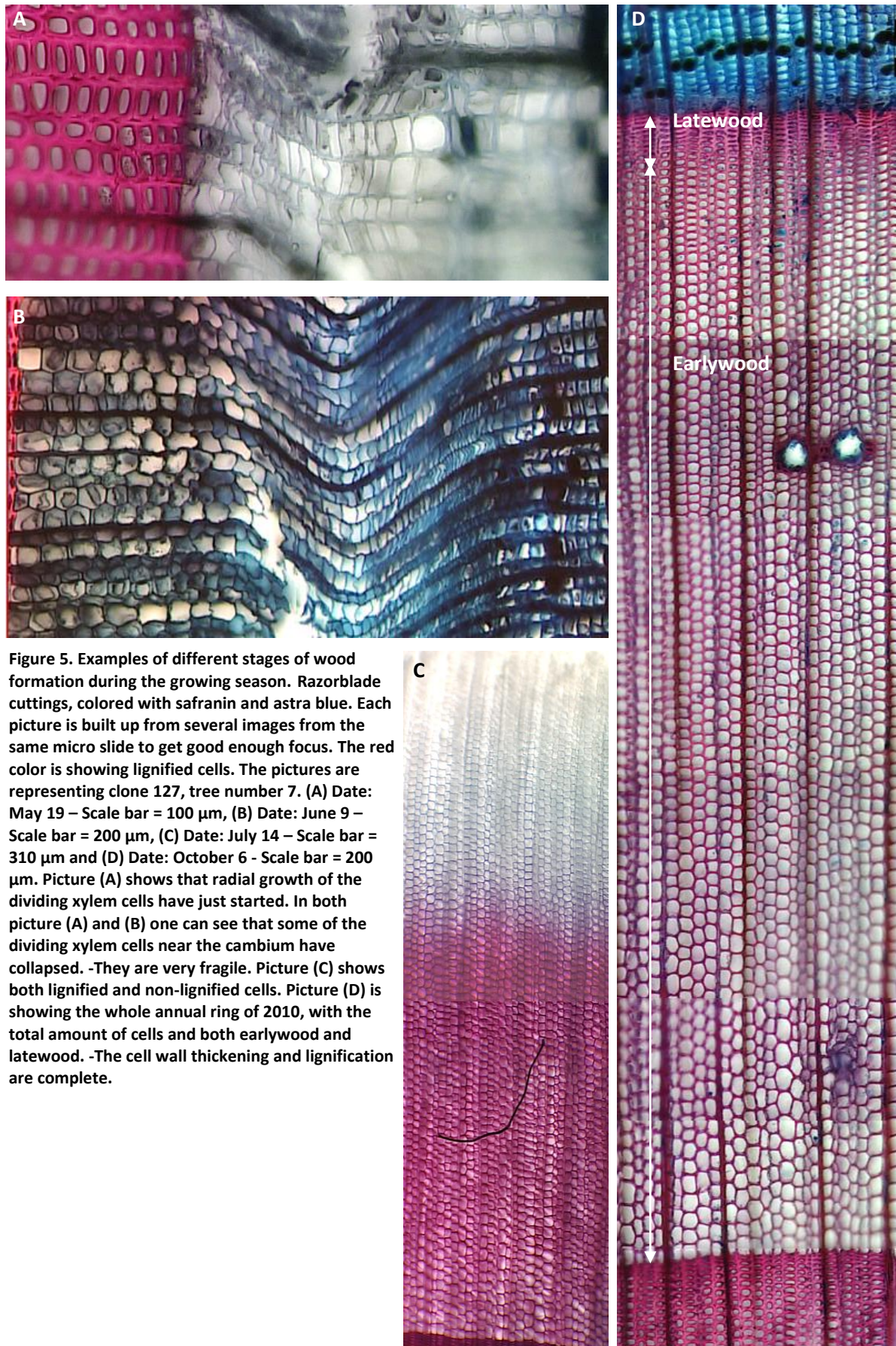
Plastic Embedding

Embedding in LR-White for Microscopy (Plastic embedding): Steps as follow: Day 1) Fixation over night, day 2) wash 15 min, dehydration 15 min: 70-, 90-, and 95 % EtOH and 15 min times four 100 % EtOH, infiltration overnight: 1 LR White : 3 EtOH, day 3) infiltration over night: 1 LR White : 1 EtOH, day 4) infiltration over night: 3 LR White : 1 EtOH, day 5) infiltration over night: 100 % LR White, day 6) embedding over night: 100 % LR White, 60°C. For sectioning the samples we used an Ultramicrotome, type: Leica EM UC6, with diamond knife. This method was tested on the samples sampled the 23^d of June. The preparation and sectioning of the first 16 micro-cores took approximately two weeks.

Razorblade Cutting

Microscope cross-sections were made by hand, using a razorblade as cutting tool. A stereo light microscope (Wild M3, Leica Microsystems, Mannheim, Germany) was used to find the right, radial direction for slicing the samples. It was important to keep the samples moist at all times to maintain the soft and vulnerable cambium in the best possible way for registration. To make the samples ready for measurements a coloring process was needed. The coloring process was done in six stages, using the colors Safranin, which stains lignin and Astra Blue (with 1 % Aqueous) which stains cellulose, rinsing in water and 96 % ethanol between the coloring stages. Coloring: Safranin, (25 sec.)

rinse in water, then ethanol, Astra Blue (5 sec.) rinse in ethanol, and finally in 0.05 M PIPES-buffer. Examples of colored razorblade cuts in different stages of wood formation are shown in figure 5.



2.3.2 Cell Counting and Measurements

As experienced in the lab at Metla, the quality of the samples was varying. In the perfect sample, definitions are easy to do. In reality, the cambial zone is often squeezed and curved, which makes it difficult to decide where the first enlarged cell is. A microscope (Leica LMD6000, Leica Microsystems, Mannheim, Germany) was used for the cell counting, measurements and picture-taking for later control. For the measurements of defining the phases of xylogenesis, it was relied on the definition rules from (Savidge 2003) and (Barnett & Jeronimidis 2003). The phases are divided in three; a-, b- and c-phase, which respectively represent radial enlargement, secondary cell wall thickening- and lignification, and mature tracheids. Dividing cells were ignored in the definition of a-phase, the first cell measured was the one which was noticeable larger than the other cells in the cambium. The stage between a- and b-phase was made by looking at the cell wall thickness and the color (If the cell corner stains red, it means that lignification has started and the phase is b), shown in figure 5. The definition between b- and c-phases was also made by looking at color differences and cell wall thickness. The mature tracheids in the c-phase should be totally red, while tracheids in the b-phase will still have some blue color showing in the cell wall layer next to the cell lumen. To make sure that the calculation of c-phase only contained mature cells, a couple of more cells in b-phase were counted since the color definition here is a little uncertain. For defining latewood tracheids, the definition according to Mork (1928) was used. The tracheids are defined as latewood if double wall thickness of one mutual cell is equal to or greater than the diameter of the cell lumen (Figure 6) formula 2 (2). To be able to compare the results with other studies such as Mäkinen et al. (2003) and Larson (1969) it was found necessary to use their interpretation of Mork's definition, which defines a latewood tracheid as if the common cell wall thickness between two mutual tracheids multiplied by two is equal to or greater than the diameter of the cell lumen, formula 1 (1). Both interpretations of Mork's definition are discussed by M.P. Denne (1989). The formulas from Denne 1989 are quoted under and visualized in figure 6.

$$(1) \quad 2a \geq b$$

$$(2) \quad 2c \geq b$$

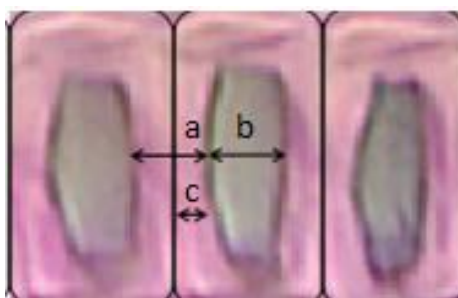


Figure 6. Together with the two formulas this figure shows how the definition by Mork (1928) is interpreted (Denne 1989): a. double wall thickness; b. lumen diameter; c. single wall thickness.

2.4.1 Statistics

All the statistical analyses were made with the program JMP® 9 (SAS Institute Inc. 2009). Significance was considered when $p < 0.05$. Microsoft Excel was used to make easier analyses and representative tables or figures to convey the results in the best way. One-way and two-ways variance analysis (ANOVA) was used to test if there were differences between clones in total number of tracheids, annual ring width, latewood percentage and start and cessation dates (Formula 3).

$$(3) \quad Y_{ij} = \text{mean} + \text{clone}_i + \text{replicate}_j + e_{ij}$$

Here clone is considered a fixed effect, while replicate and e (residual) is assumed to be random normal distributed with mean 0 and respective variances (σ^2)

In case of significance in the ANOVA test, a Tukey-Kramer and HSD test was used to identify which clones that were the statistically different.

A variance analysis was used to test the differences in cell formation between the early and the late clones. We have been focusing on using the average results for each clone when presenting the results.

The total number of tracheids was found for each tree as the average of the amount of tracheids measured at the end of the growing season, when development of new tracheids had ceased. That is to say, when there were no more new cells in the a-phase. The dates of initiation of latewood formation and the date for cessation of growth for each tree was determined from nonlinear regression curves between number of formed cells as the independent variable and number of days from January first as the explanatory variable. The curves were fitted with a Weibul four parameter model. In Formula 4 the model is solved with respect to x to find the different dates for initiation of latewood formation.

$$(4) \quad x = e^{\frac{\ln\left(-\ln\left(\frac{\theta_1 - y}{\theta_2}\right)\right) - \theta_3}{\theta_4}}$$

When defining the date for latewood formation, the y variable is the number of cells at the initiation point of latewood formation. To find the number of cells, the mean number of measured latewood cells at the two last sampling dates were used and subtracted from the mean total of tracheids at cessation of growth. The date for cessation of growth was confirmed by the fitted curve from «Weibul», in the intersection point at 99.5 % of the horizontal top curve where it was flattening out. Thus 99.5 % of the total number of cells was set as x to find the date.

Annual ring width and latewood percentage was measured as an average from the two last sampling dates in October, as the growth presumably would be finished at this stage. Using the microscope and photoshop these parameters were measured with accuracy at micro-meter level.

The family structure partly complicates the statistical analysis, as the clone 320 and 323 originates from the same full-sib family (Table 3). This is unfortunately ignored.

3. Results

3.1 Diameter and Height

3.1.1 Diameter at Breast height and Height

No significant differences in diameter at breast height or height were found between clones ($p>0.05$). Mean diameter at breast height was approximately 146 mm, and total average tree heights were 12.6 m. The mean diameters at breast height for each clone; 127: 150 mm, clone 320: 135 mm, clone 323: 136 mm, and clone 337: 163 mm.

3.2 Bud Flush and Shoot Development

3.2.1 Differences between Clones in Bud development

The buds had already started to swell slightly on May 26th, buds were measured from the top bud development. Growth rhythm between the clones was as expected (Figure 7), with the clone 337 representing a very early bud development in the spring and clone 127 representing a very late bud development. Clone 337 was significantly earlier in bud development than clone 127 from day 33 – 47, and also significantly earlier than «late» clone 323 (Figure 8), Two-way ANOVA followed by Tukey-Kramer test, f-values from 6.35 – 12.89, $p<0.05$. One case of significance in bud development between clone 320 (early) and 323 (late) were found for day 33, so the characteristics of the known apical growth rhythm were there (Figure 7) and (Figure 8). Clone 127 was also significantly later out in the stages of bud development than clone 320.

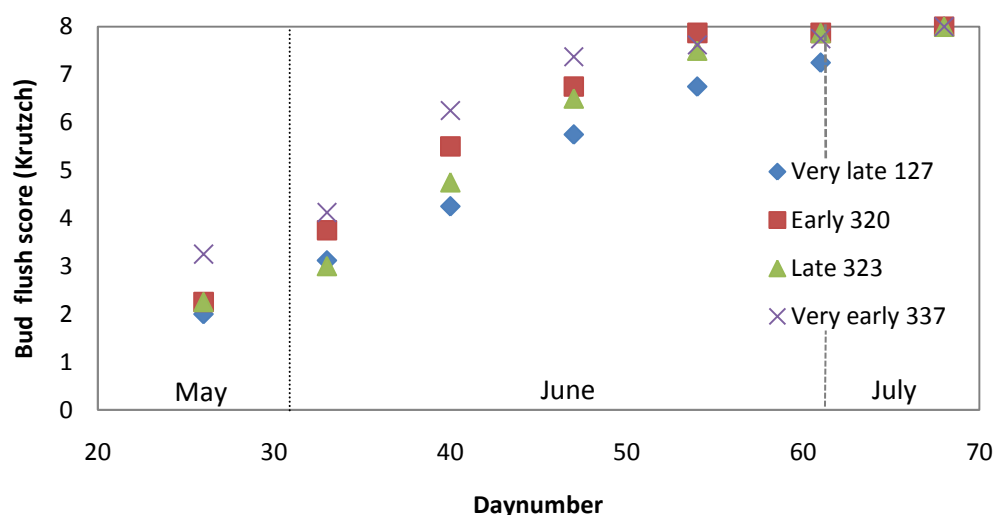


Figure 7. Bud flush score (Krutzch), from 26th of May. All the clones had finished apical top growth at day 68, which is 7th of July.

May 26th, clone 127 still had one out of four trees with dormant buds. The other clones all had slightly swollen buds or were in the classification of «burst of bud scale» as in clone 337. The same tendency was seen the next following weeks, with an increase of one level every week. Clone 127 was consistently later than the other clones, however all the clones had got one tree with the classification «all needles more or less spread» on the 23^d of June. As of July 7th, all the clones had finished the bud developing process.

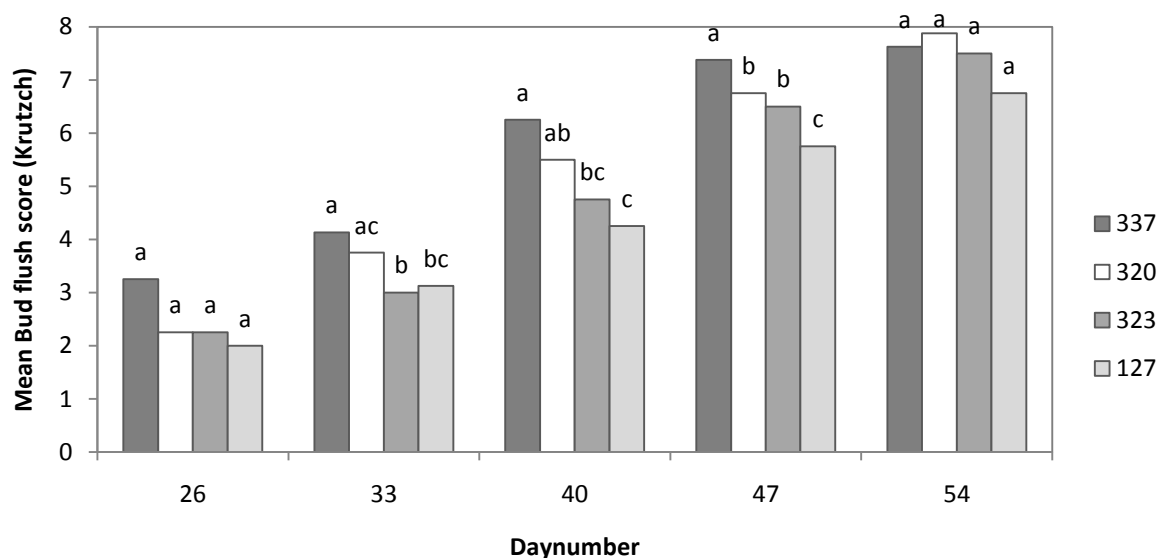


Figure 8. Mean bud flush score (Krutzh) for day number. Levels not connected by same letter shows significant differences of bud flush score between clones, $p < 0.05$, $N = 16$.

3.3 Cessation of Tracheids in Different Phases

3.3.1 Onset of Cell division in Cambium

The date for onset of cell division in the cambium was approximately from 12th of May till 19th of May, calendar week 19 and 20. However there was one tree within three of the clones, which did not start before 26th of May (Appendix 1). There was no significance between the early or the late flushing clones in onset of cell division (Table 5).

Table 5. Onset of cell division in cambium. No significantly differences between clones. p -, f - and n -values from ANOVA.

Clone	Mean, Day 12 th May	Mean, Day 19 th May
127	0	2
320	0.25	2.75
323	0.25	1
337	0	1.75
$p = 0.59$, $f = 0.67$, $n = 16$		$p = 0.48$, $f = 0.85$, $n = 16$

3.3.2 Phases of Wood formation

There were some significant differences between clones in the different phases of wood formation, from cell division and number of divided cells, enlarging xylem cells to maturing and lignified cells.

The late flushing clone 127 appeared to be the one most different from the other clones, and did produce significantly more cells during the whole growth period in the two b- and c-phases of wood formation, and as follows also in total number of tracheids at the end.

The clones had tracheids developing in b-phase, from week 22, and the lignification of maturing cells had started slightly in week 23 and 24 (from 9th of June). The first fully lignified and completed secondary cell wall formation were found from week 24 (16th of June).

Nearly no Significance between Clones in a-phase

In general there were only small and non-significant ($p > 0.05$) clonal differences in number of cells in the a-phase, dividing and radial enlargement cells. Only for one date, the 11th of August (day number 103) clone 127 showed to have significant more cells in the a-phase, than clone 323 and 320 (Figure 9) and (Table 6).

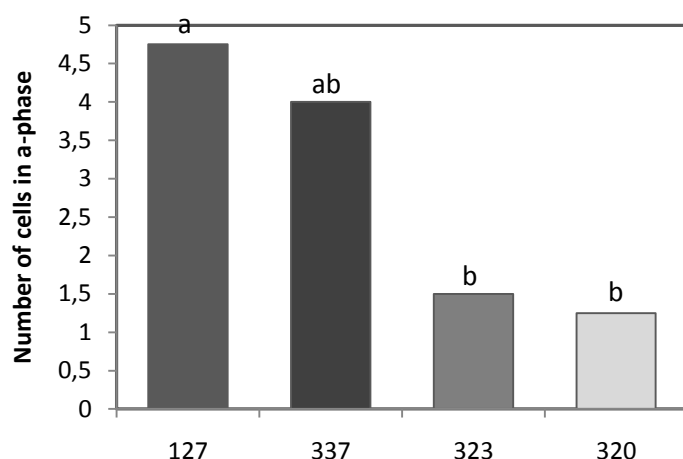


Figure 9. Numbers of cells in a-phase for clones. Significantly more cells in a-phase for clone 127 at 11th of August. Levels not connected by same letter are significantly different.

Table 6. Overview of which day numbers of the growth period that shows to have significant and no significant differences between clones in numbers of cells produced. Two-way ANOVA (Formula 3), sort was tested against residual and degrees of freedom were 3 in numerator and 8 in denominator. N = 16. Significance is shown for $p < 0.05$, and highlighted in grey.

Day number	Date	Number of cells in the different phases							
		A-phase		B-phase		C-phase		Total	
		f-value	p-value	f-value	p-value	f-value	p-value	f-value	p-value
12	12 May	0,98	0,45	-	-	-	-	0,98	0,45
19	19 May	1,06	0,42	1,00	0,44	-	-	1,01	0,44
26	26 May	1,06	0,42	1,63	0,26	-	-	0,89	0,48
33	2 June	2,28	0,16	3,95	0,053	-	-	2,92	0,10
40	9 June	2,01	0,19	1,25	0,35	-	-	1,33	0,33
47	16 June	2,75	0,11	3,66	0,06	0,83	0,52	4,85	0,03
54	23 June	2,14	0,17	2,32	0,15	5,09	0,03	2,95	0,10
61	30 June	1,19	0,37	1,47	0,29	8,28	0,01	3,22	0,08
68	7 July	0,21	0,88	2,95	0,10	5,52	0,02	4,28	0,04
75	14 July	1,60	0,26	2,97	0,10	11,92	0,003	10,04	0,00
82	21 July	2,09	0,18	7,83	0,01	3,43	0,07	5,82	0,02
89	28 July	0,87	0,50	6,33	0,02	11,26	0,00	13,56	0,002
96	4 Aug.	1,38	0,32	1,27	0,35	5,16	0,03	6,40	0,02
103	11 Aug.	7,38	0,01	1,23	0,36	6,92	0,01	7,26	0,01
110	18 Aug.	2,10	0,18	5,19	0,03	2,60	0,12	3,37	0,08
117	25 Aug.	2,47	0,14	5,50	0,02	5,90	0,02	7,26	0,01
124	1 Sept.	2,00	0,19	4,64	0,04	6,75	0,01	6,78	0,01
131	8 Sept.	3,28	0,08	4,24	0,045	5,00	0,03	4,17	0,047
137	14 Sept.	1,00	0,44	5,52	0,02	3,36	0,08	4,70	0,04
145	22 Sept.	-	-	9,98	0,004	6,27	0,02	7,95	0,01
152	29. Sept.	-	-	2,23	0,16	12,57	0,00	10,75	0,004
159	6 Oct.	-	-	1,40	0,31	3,78	0,06	3,18	0,08
166	13 Oct.	-	-	0,21	0,89	4,68	0,04	3,93	0,054

Significantly more Cells in the b-phase for Clone 127

In the b-phase, cells in secondary cell wall thickening- and lignification, clone 127 did have significantly more cells than clone 323 for the dates; 21st – 28th of July, 25th of August – 1st of September and 14th of September, than clone 320 for the dates; 21st – 28th of July and from 18th of August – 22nd of September, and than clone 337 for the dates; 18th of August and 14th – 22th of September (Table 6). Also clone 337 did have significantly more cells than clone 323 for the date; 22nd of September, and than clone 320 for the dates; 21st of July, 25th of August and 22nd of September. Clone 320 showed to have significantly more produced cells than clone 323 at 22nd of September in the b-phase ($p < 0.05$). Table 6 shows the day numbers and dates for both significance and no significance between the clones, Table 7 under shows for which clones the significance

applies. It is essentially clone 127 that stands out. Note that the differences were not significant early and late in the season (Table 6).

Table 7. Significant differences between clones in the b-phase, $p < 0.05$.

B-phase:			
Day number	Significances:		
82	127 > 320, 323	337 > 320	
89	127 > 320, 323		
110	127 > 320, 337		
117	127 > 320, 323	337 > 320, 323	
124	127 > 320, 323		
131	127 > 320		
137	127 > 320, 323, 337		
145	127 > 320, 337	337 > 320	320 > 323

Significance between Clones in c-phase

The significance in total number of cells in c-phase, mature tracheids, appeared at the following dates, from Table 6; Clone 127 did have significantly more tracheids in the c-phase than clone 323 from 23th of June – 14th of July, 28th of July – 11th of August, from 25th of August till 8th of September and from 22nd – 29th of September. Clone 127 also had significantly more cells than clone 337 (28th of July and 8th of September) and clone 320 (14th, 28th of July – 11th of August and 1st – 29th of September). Clone 320 showed to have significantly more cells than clone 323 at 23th – 30th of June, and clone 337 showed to have significantly more cells than 323 from 23th – 30th of June, 14th and 28th of July and 11th of August, and than clone 320 at 29th of September. See the list over significant results in Table 8.

Table 8. Significant differences between clones in the c-phase, $p < 0.05$.

C-phase:			
Day number	Significances:		
54	127 > 323	320 > 323	337 > 323
61	127 > 323	320 > 323	337 > 323
68	127 > 323		337 > 323
75	127 > 320, 323	320 > 323	337 > 323
89	127 > 320, 323, 337		337 > 323
96	127 > 320, 323		
103	127 > 320, 323		337 > 323
117	127 > 323		
124	127 > 320, 323		
131	127 > 320, 323, 337		
145	127 > 320, 323		
152	127 > 320, 323		337 > 320

Mean total number of Tracheids

Figure 10, show the clonal mean number of tracheids formed at each sampling date during the whole growing season. From the analysis of the different phases of wood formation above (Table 6) and (Table 9) clone 127 produced a significantly higher number of tracheids in total ($p < 0.05$) mainly due to a higher rate of cell divisions in June and July (Figure 10) and (Figure 13). In late July and middle of August the total number of new formed tracheids was decreasing. As seen from Figure 11, the variation in number of tracheids was big between trees within the different clones. The total variation is shown with error bars for each sampling date. The variation in number of cells between trees within clone 337 was larger compared to the variation in the other clones (Figure 11).

Table 9. Significantly differences between clones when taking mean total number of cells for each sampling date into account, $p < 0.05$.

Mean total number of tracheids:		
Day number	Significances:	
47	127 > 323, 337	
68	127 > 323	337 > 323
75	127 > 320, 323	337 > 320, 323
82	127 > 320, 323	337 > 323
89	127 > 320, 323, 337	337 > 320, 323
96	127 > 320, 323	
103	127 > 320, 323	337 > 323
117	127 > 320, 323	337 > 323
124	127 > 320, 323	
131	127 > 320, 323	
137	127 > 320, 323	
145	127 > 320, 323, 337	
152	127 > 320, 323	337 > 320

A significant difference in the mean total number of produced tracheids at the end of the growth period, when taking the average from when no cells were found in a-phase, was found between clone 127 and clone 323 and 320 (Figure 12). Two-way ANOVA followed by Tukey Kramer.

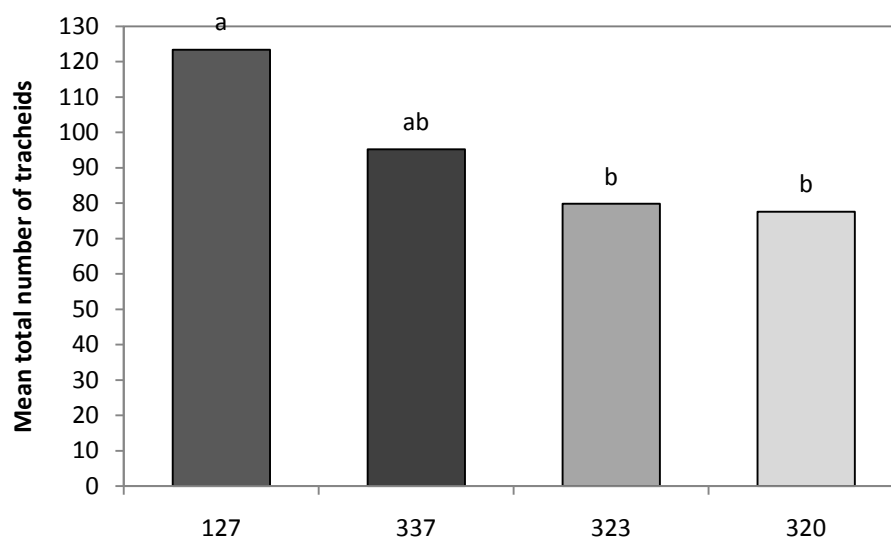


Figure 12. Mean total number of tracheids for the four Norway spruce clones. The two-way ANOVA followed by Tukey-Kramer test shows significances between clone 127 and 323 and 320. Levels not connected by same letter are significantly different.

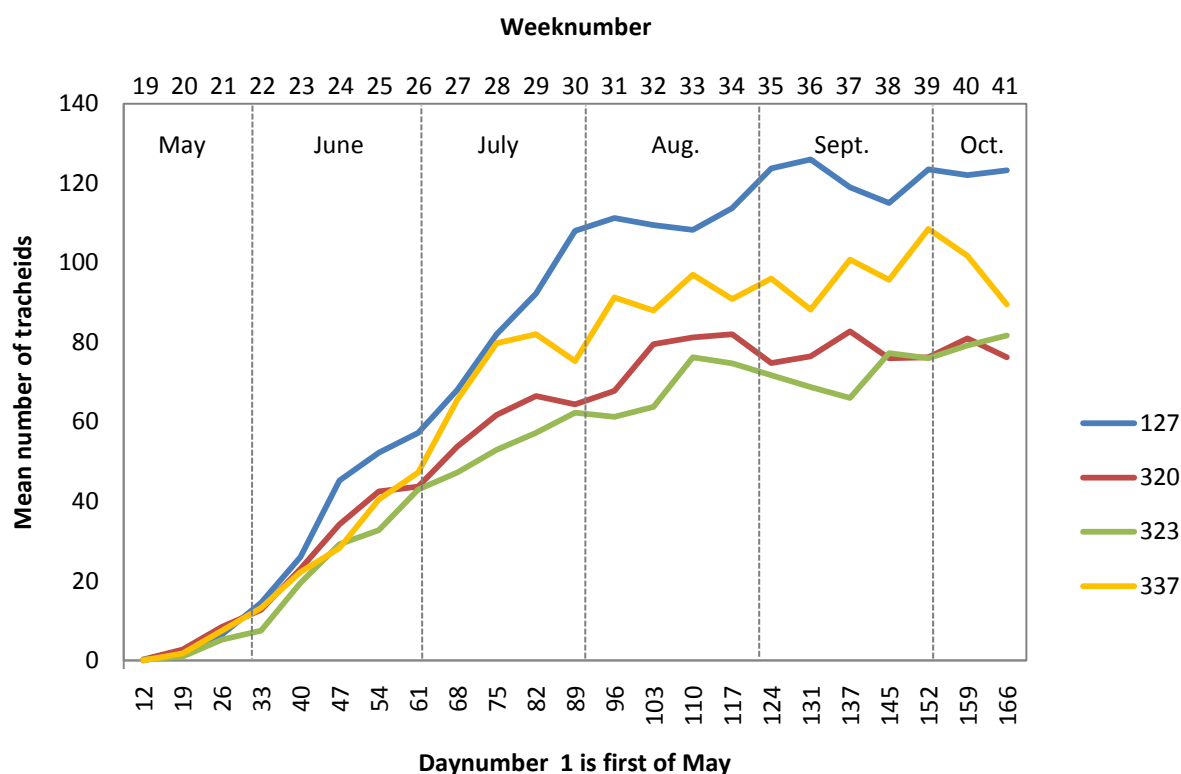


Figure 10. Mean number of tracheids produced per clone for each sampling date, from May until October. Day 1 is first of May. Clone 337 – «very early», 320 – «early», 323 – «late» and 127 – «very late».

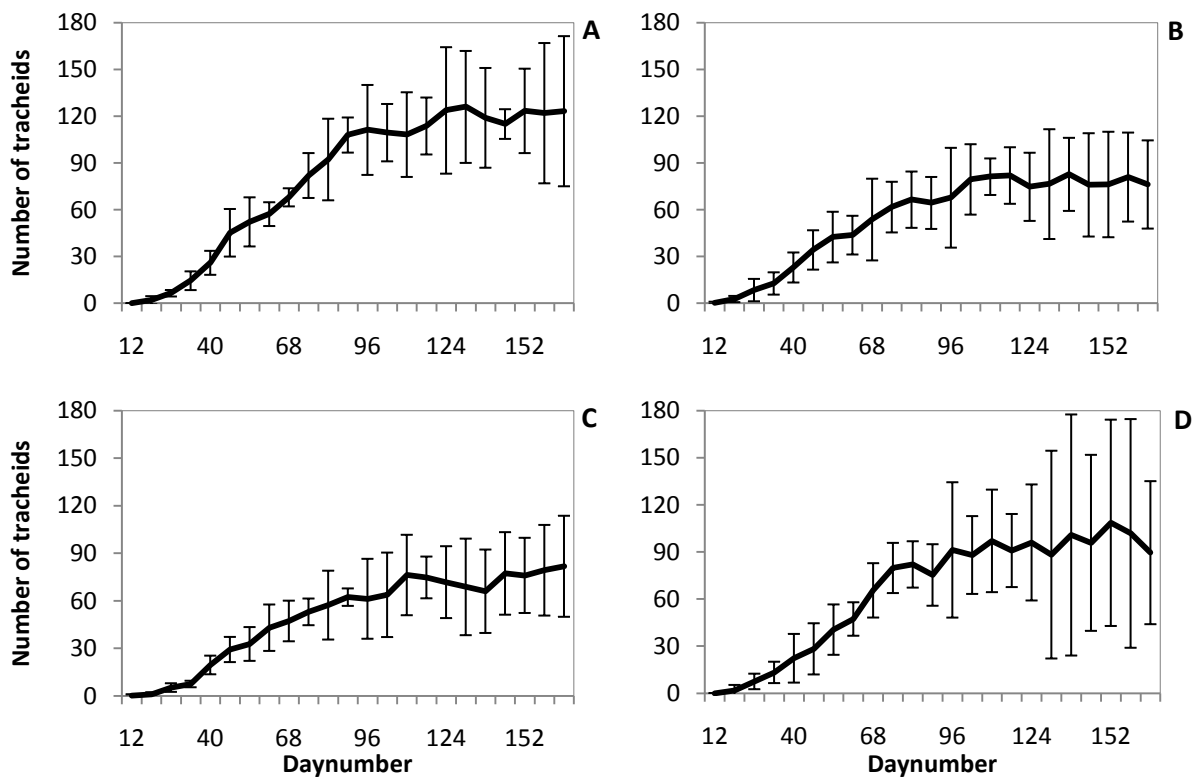


Figure 11. Mean value of number of tracheids produced per clone (A-D) on each sampling date. The total variation inside each clone is shown within the error bars for each of the days. (A) clone 127, (B) clone 320, (C) clone 323 and (D) clone 337.

3.4 Relationship between Apical growth hythm and Wood formation

A trend in most dividing cells for the early clones at the start

For day 26, the two early bud flushing clones had got the most cells in a-phase (Figure 13). However the difference was not significant. (Variance analysis for cells in a-phase, day 26 with random effects for replicates). The early clones did produce the most cells in a-phase until day 33 (Figure 13).

Significance in bud flush score and wood formation at day 47

From day 33 and 40 it was found that very late clone 127 produced more cells than the other clones (Figure 13) and (Figure 14). At day 40, there was approximately 1.5 week of difference in growth between the earliest ant the latest clone when looking at number of produced cells. From day 47 clone 127 had more cells in the a-phase than the others. For the day numbers, 33, 40 and 47 which showed significance between clones in bud flushing, there were also only significances in phases of wood formation at day 47 for mean total number of produced tracheids. This applied only to clone 127, which showed to have significantly more tracheids than clone 323 and 337 (Table 9). From figure 14, one can see that the trend is that the «very early» and the «very late» clone have got the most formed tracheids when reached the end of the Krutzch scale (all the needles more or less spread). There is a relationship between apical growth rhythm and wood formation, but this relationship is not determined by the variation of earliness in the known apical growth rhythm. Date of initiation of apical growth was related with the initiation of wood formation in the spring. The finish time for apical growth in July could also be related to the initiation of latewood formation.

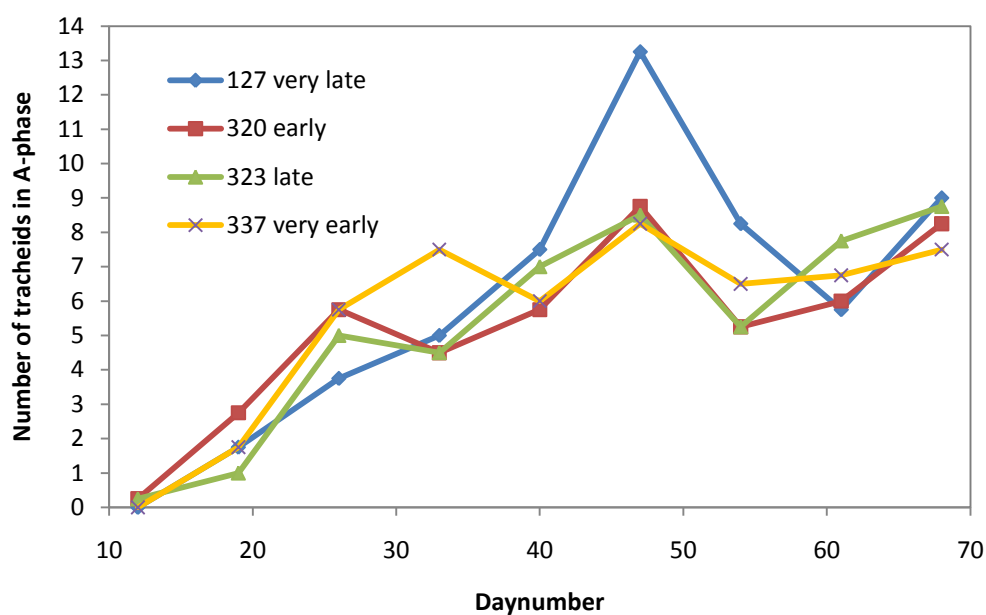
Figure 13. Number of tracheids in A-phase

Figure 13. Number of divided cells in A-phase, from the beginning of May until the beginning of July, when the buds are formed.

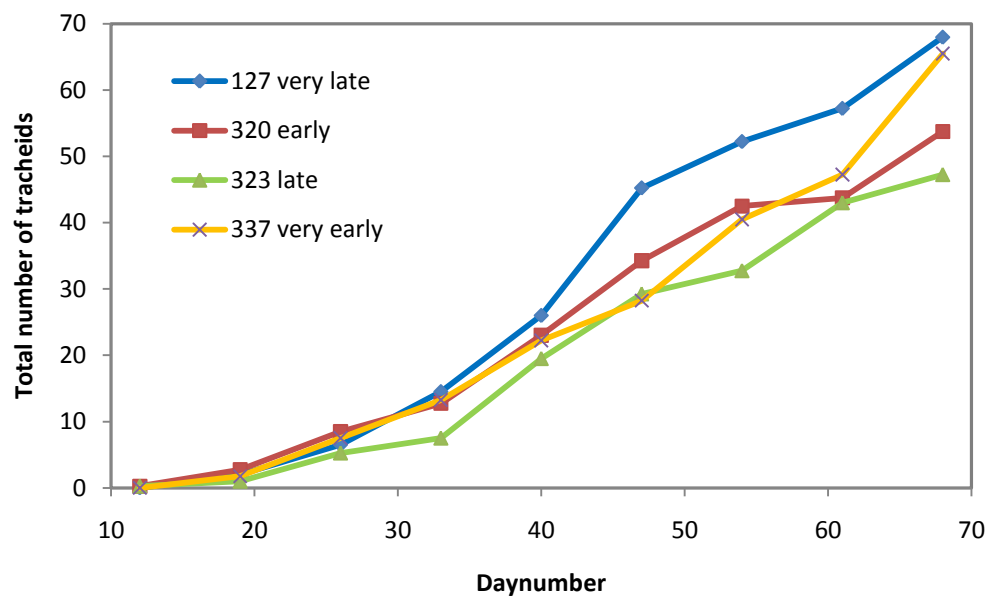
Figure 14. Mean number of tracheids in the stage of bud development

Figure 14. Total number of tracheids from the first part of the growing season when the buds are formed. From beginning of May (12th) until the beginning of July (7th).

3.5 Initiation of Latewood Formation

According to Mork's definition, formation of latewood occurred in the period from 28th of July until 8th of August (with the mean date per clone; 320: 28th of July, 337: 30th of July, 127: 2nd of August, and 323: 8th of August). The second blue line from left in figure 15 shows the date for initiation of latewood for each clone. Within clones the date for initiation of latewood formation varied from 4th of July until September 11th. The late clones, clone 323 and clone 127 are also latest out with the latewood formation. Using formula 1, the initiation date was set to be about one to two weeks earlier, from 17th until 26th of July (with the mean date per clone; 320: 17th of July, 337: 22nd of July, 127: 25th of July, and 323: 26th of July). Within clones the dates varied from June 30th to August 30th. There was no significance of the date of initiation of latewood formation between clones, by using either of the formulas ($p > 0.05$). The blue line to the right in figure 15 shows the date of the end of cell division. There were no significant differences between clones when testing for $n=16$, but from Weibuls model for each clone in total, late flushing clone 323 showed to end much later.

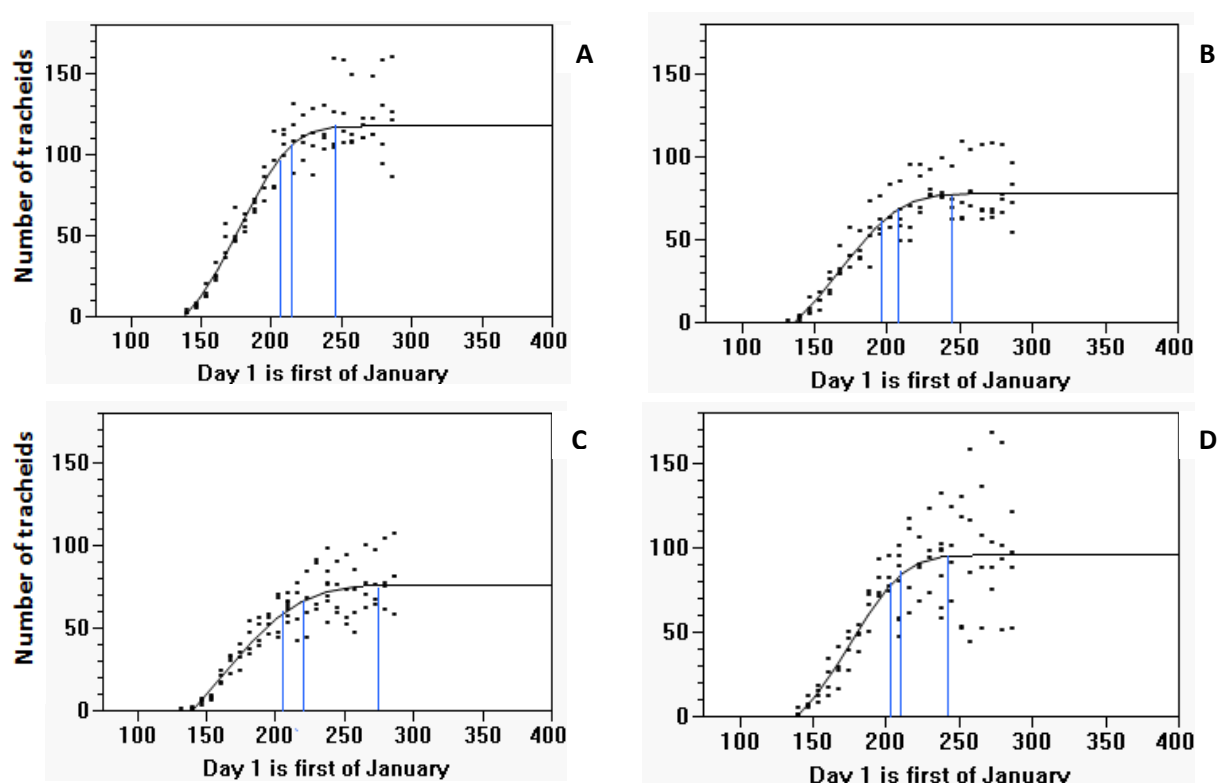


Figure 15. Dates for initiation of latewood formation for the four clones, A: 127, B: 320, C: 323 and D: 337. Number of days from 1st of January, day 132 is 12th of May. The first two respective lines from the left are showing the dating by formula 1 ($2a \geq b$) the interpreted definition of latewood and formula 2 ($2c \geq b$) the definition by Mork (1928). The blue line to the right shows the date of the end of cell division.

Visually, it was not possible to see the distinction between earlywood and latewood before the first week of September, calendar week 35 and 36. Preformed latewood cells were visible from week 36 – 38. The lignification was not done before the last week of measurements in October, week 41. This was according to the pictures taken with a microscope.

3.5.1 Cessation of Growth

The timeframe for when the clones stopped producing any new cells was from mid-August to mid-September on tree individual level. The mean date for each clone based on the Weibul model was respectively from 30th of August to 1st of October (clone 127: 1st of Sept. clone 320: 1st of Sept. clone 323: 1st of Oct. and 337: 30th of Aug.). With the «Weibul» profile and the determination at 99.5 % the total spread between trees gave a large effect on tree individual level.

Only one clone appeared to have completely finished growth at the end of the sampling period, 13th of October. The following trees were likely not to have finally completed wood formation in the secondary cell wall at the end of the sampling period; Clone 320, tree number 1, 12 and 16, clone 127, tree number 3, and clone 337 with tree number 9. There was still some blue color showing in the last couple of tracheids in these trees annual rings. None of the sample trees were registered to have developed lamma shoots at the autumn.

3.5.2 Annual ring width and Latewood percentage

There were no significant differences in the latewood percentage and annual ring width between clones (Oneway ANOVA, Tukey-Kramer test with p-value = 0.58, f-value = 0.67, n = 16). The two clones with the «late» definition did have the smallest annual ring width (Figure 16). They also had the greatest amount of latewood, though this was not statistically significant. Between the two «definitions» of latewood in figure 16A and B one can see that there is a certain difference in the amount of latewood.

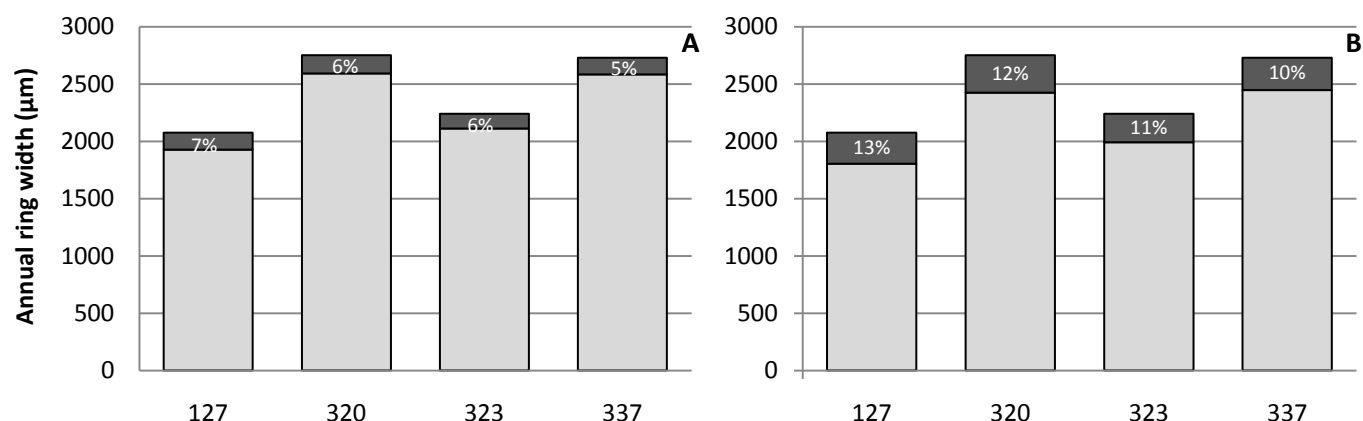


Figure 16. Annual ring width (μm) and latewood percentage of the four Norway spruce clones. Definition of latewood by Mork 1928, interpreted by Denne (1989) formula 2 (A) and interpreted definition of latewood by Denne, formula 1 (B).

3.6 Compass directions

3.6.1 Variations in Cell number and Ring width around the stem

When sampling from all four compass directions the highest amounts of tracheids were sampled in the north and west directions, and the lowest amounts of tracheids were recorded in the south and eastern directions. The number of measurements from the different compass directions were however insufficient to run any statistical analysis. The differences from one side to another were at the most 30-40 tracheids.

4. Discussion

4.1 Material and Methods, Limitations

4.1.1 Study material

The study material contained only 16 trees in total, from 4 different clones. With only 4 trees (ramets) representing each clone, the mean values and particularly the variation within each clone was estimated unbiased but with a considerable random error. Few replications give low degrees of freedom in the statistical tests used, and thus the differences between the means need to be relatively big to show significance.

The measurements took place during only one growing season, which is another weakness, because of the possibilities of getting data from unusual growing conditions. The most desirable study would be to have more replicates and measurements within clones, over several years. However this was not possible with the resources and time available.

The family structure of the selected clones was considered to be of secondary importance as the aim of this study was to test whether early and late bud flush could have any impact on timing of xylem formation. The clones selected were chosen on basis of their known and contrasting apical growth rhythm, and it was desirable that they had the same width and height to get as equally comparable data as possible.

4.1.2 Sample Preparation

Three different methods for sample preparation was tested, paraffin embedding and plastic embedding with subsequent microtome cutting and at last razor blade cutting. The embedding methods, especially the plastic embedding required quite a lot of equipment and they were both quite comprehensive and more time-consuming, than the simple razorblade method. A relatively inexperienced person would be able to prepare and analyze 16 micro-cores a day, as of the paraffin embedding method; an experienced person would be able to prepare approximately 10 samples a day (and the analysis would be in addition). About the same period of time would apply to the plastic embedding method. The reason why we could find the razorblade method sufficient while others prefer more complicated methods may be due to the technology and high quality of our microscopes.

4.1.3 Statistics

It is debatable whether the use of Weibul's four parameter model was optimal when calculating the dates of initiation of latewood formation and cessation of growth. Still it proved to fit the quite divergent individual trees relatively well, for the actual periods of initiation of latewood formation and growth cessation. Other methods that could be used to determine the growth are e.g. the Gompertz function (Mäkinen et al. 2008).

4.1.4 Field work

The measurement of bud flush started after the initiation of bud flush in the forest but this was not decisive for the results.

4.1.5 Compass directions impact on Xylem formation

The micro-core samples were first taken from the south side of the tree, then clockwise around the stem. Mäkinen et al. (2003) found different number of formed tracheids for different azimuths. They found significantly more tracheids on the north side of the sample tree. The lowest amount was found on the south side. Even with few trees measured, the results from this study confirm these findings. It was found that the difference from one side to another could be up to 30-40 tracheids. Still other studies, such as Liese and Dadswell (1959) refer to a greater width on the sunny side of the stem. The large difference between azimuths, and the variation in stem diameter is most likely a good explanation for the big variation found in number of tracheids measured in the late growth period, both within and between trees. A large variation between compass directions has been found to occur, especially in the autumn when the tracheid formation has ceased (Mäkinen et al. 2003). The same tendency was found in this study.

As the number of cells and tracheids varied somewhat around the stem, the ring width measurements should either have been done from the same compass direction or with total random direction to avoid differences in ring width and number of cells this may have led to. In this study the ring width was based on the two last cores taken. According to Forster et al. (2000) it is necessary to take several cores at the same time to establish more precise value for mean growth. However such a method was impossible to accomplish in this study, since cores had to be taken at least once a week during the whole growing season. For our relatively small diameter trees it was not enough space to take the number of cores Forster's method requires. To make a correction for the variation between azimuths it is still possible to add azimuth as a covariate in the analysis.

To determine the significance of compass directions on the the annual ring width and number of tracheids, Mäkinen et al. (2003) made an analysis of the effects of the compass directions, and then

took an adjustment according to this. The intention was to take this into account, since the collection of samples did provide opportunities for this.

Human error and lack of experience

It may be convenient to take into account that the undersigned is inexperienced by making the measurements required in the study. More experienced researchers may have measured things differently. The analysis results are in general influenced by systematic and random errors, and some internal quality control was made to reduce this.

4.2 Results

From the material studied, there were no clear relationships between timing of bud flush and the initiation or rate of wood formation early in the growing season. However later in the growing season, significant differences were found between clones in number of tracheids formed.

Particularly one clone was standing out; this was the «very late» flushing clone, 127. This clone formed significantly higher number of tracheids, but they were narrower and the ring width was also lower. These findings do probably not have any connections with the genetic variation in bud flush. Initiation and cessation dates for latewood formation did not differ significantly between clones. Neither did annual ring width nor latewood percentage.

4.2.1 Discussion of the Results

When discussing the results, the clones which represents one very early (337), one very late (127) and two intermediate ones (320 and 323), will respective be referred to as «early», «very early», «late» and «very late» in the text.

Bud flushing started in the middle of May. The «very late» clone was later than the other clones in the stages of bud development. This could be significantly shown in the first period of June, when the apical growth was at the largest. The «very early» clone was fastest when it came to the bud stretching. 7th of July all the clones had reached score number 8, all needles more or less spread, and were close to fully formed.

The onset of cell division in cambium happened in early- and middle of May, there was no significant relationship between the «earlier» or the «later» clones in onset of cell division. This may be related to the late and sudden start the actual spring. Poor relationships between the timing of bud flush and timing of xylem formation was found. On the other hand at 11th of August, close to the end of wood formation, the «very late» clone did have significantly more dividing cells than the two more intermediate clones. Together with the «very early» clone these two clones had got the most

dividing cells from 11th of August until the end of cell formation in middle of September. The number of dividing cells in the a-phase rapidly decreased at the end of July for all clones. Hence, cambial cell division seemed to end at the end of July in this material at this particular site.

There were thus no benefits having an early bud flush when it came to the numbers of dividing and radial enlargement cells in the a-phase early on in the growth period. The two early flushing clones tended however to form more cells at the first sampling dates, but the differences in the means were not significant. It seems to be a weak or nonexistent genetic link between the clones ranking of earliness and wood formation in the spring. Therefore, it most probably is not the same genes that control apical and cambial growth rhythm. Nevertheless, Larson's (1969) theorize, from general findings, that the formation of earlywood happens from when the tree is elongating in the spring. Worrall (1970) found it also to be a positive correlation between the date of initiation of radial growth at breast height and the date of initiation of height growth.

The results showed that there were clones with significantly more cells in the phase of secondary cell wall thickening and lignifications, which mainly appeared in the second half of the growth period. One clone did especially stand out; this was the «very late» one, which generally showed to have higher number of tracheids than the other clones. However, periodically, the «very early» clone showed to have significantly more cells than the two middle ones. In the phase of fully lignified and finished maturing xylem from 23th of June and until the end of the maturing process, the «very late» clone were the one most different from the other three clones. And throughout the whole period, it showed to have significantly more tracheids than the classified «late» clone, see figure 10.

As described by Zimmermann (1964), the requirements for cambial activity is temperature, growth regulators such as, auxin especially, supply of mineral nutrients, carbohydrates and nitrogen substances and sufficient water to maintain cells in a turgid condition. Components of tracheid size like length, cell wall thickness and lumen diameter is also influenced by temperature, light intensity and daylength in some varying degree (Zimmermann 1964). It is apparent that environmental factors play a decisive role in wood formation; however in this study the clones did have almost the same growth conditions. This makes it reasonable to believe that there are other genes within these clones that control the number of divided and formed tracheids. It is found from former studies that there are genetical variations in tracheid size and number of formed tracheids (Hannrup et al. 2004). Cambial activity is explained by Larson (1961, 1963) to cease when the supply of auxin is reduced because of cessation in shoot and leaf growth (Zimmermann 1964).

The number of formed cells fluctuated greatly from date to date, also within trees, especially at the end of the growth period. Clone 337 showed to have the greatest variation in number of tracheids

measured within and between trees. Some of the variation between trees might have something to do with the variation in number of cells with compass directions. It might also have something to do with the differences in light conditions for the single trees.

At the end of the growing season it was still the «very late» clone which showed to have the highest number of tracheids. But, due the limitations of the material, only four clones, it cannot be generally concluded that late flushing individuals form more tracheids. The study also shows that the number of tracheids is probably influenced by other genes than which were controlled in the trail from where this material was taken from.

It is interesting to see the fact that «early» clone 320 and «late» clone 323, which is full-sibs seemed to have a fairly parallel development and fluctuations in the wood formation (Figure 10). The trend was also that «early» clone 320 was consistently later in all phases of development compared to the «late» clone 323. The parallel fluctuations could be due to a common response to environmental, e.g. climatic, influences during the growing season. As the clones are full-sibs, they share 50 % of the genes.

Latewood formation occurred from mid-July until early August. This matches pretty well the findings by Worrall (1970) who found the date of initiation of latewood deposition to vary from June 30th to August 13th, with a mean of July 14th. He also found that the date when the height growth ceased correlated with the initiation of latewood deposition (Worrall 1970). The late flushing clones were slightly later out with initiation of latewood formation than the earlier ones. From the results presented in figure 16 the two late flushing clones did slightly have a greater or similar percentage of latewood than the other clones. They also had a smaller annual ring width, for the year when the micro-cores were taken (2010), than the early flushing clones. This is opposite to what was the case for DBH, where the latest clone had about the largest DBH. However, the differences measured in annual ring width and DBH between the clones were not significant. Hylen (1997) explained the variation found in wood density traits probably was due to of differences in flushing time between the families. E.g. Worrall (1970) and Dietrichson (1964) found that late flushing trees had a lower wood density than those flushing early (Hylen 1997). The results from the present study do not confirm these findings. A possible explanation, which also was reported by Hylen (1997), is that latewood density and latewood percentage appear to be under stronger genetic control than overall density.

There are hence differences in cell expansion between clones. Clone 127 did have the most tracheids produced at the end of the growth period, while the annual ring width was smallest. It is so that density in general decreases with increasing annual ring width (Bowyer et al. 2003). In addition to a

large variation in density between species there are also variations within each tree, between trees in the population, between populations, between regions, climate, latitude and altitude. Plant spacing and site quality has also much to say. Larger plant spacing, longer branches and deeper crown which leads to more needles gives a greater ring width and lower density.

Cessation of growth happened from late August until 1st of October, but no significant differences were found between clones.

The last sampling date, 13th of October, only clone 323 appeared to have completely finished cell wall development. Referring to former studies, tracheid cell wall development is likely to continue until October although cambial cell division usually ceases in late August or September. In some cases the tracheid cell wall development also resumes the following spring (Larson 1994). Even «Late» clone 323 was the only clone that appeared to have finished the cell wall development completely it ended the cell production later than the others. It also had a much lower total production of tracheids. Still the cessation of latewood was the same as for the rest.

4.2.1 Impacts by Exogenous factors

Precipitation and Temperature

The precipitation during the growing season of 2010 was close to normal and no large fluctuations in the temperature, referring to the weather data (Figure 3) occurred. It was thus no droughts or frosts that may have had any substantial impact on the results.

However the spring of 2010 was relatively late which could be part of the reason why the start of cell division in cambium was relatively similar for all the clones. The trees need a certain temperature sum to activate the growth, and in this case the temperature was substantial in a way that they all got the necessary temperature sum within a few days. If the temperature had gradually increased from April as normal, we probably would have seen somewhat other results, and differences between the early and the late clones, both when it comes to flushing and wood formation. The trees react to heat in the spring while in the autumn they react to light and day length. Temperature is the most important factor for the onset of xylem formation (Partanen et al. 2001), but the onset of growth is also controlled by photoperiod, water availability and auxin production (Larson 1967).

Competition

The sample trees did approximately have the same height and diameter at breast height, so there were no cases of suppressed individuals for the trees investigated. Thus competition most probably had no direct effect on the results.

4.2.2 Practical Implications of the Results

In further selection of breeding material, this result does not indicate any implications by using late flushing materials, when it did not have any significant negative impact on the wood formation. It looks like there are different genes that control some of the wood formation and the case with earliness. As follows it may occur possibilities to find trees positive with respect to growth, growth rhythm and wood properties.

Just to name something, in some parts of the paper industry, it may be advantageous with numerous and narrower tracheids as shown in one of these particular clones, because it may give a flatter and stronger paper. From the material of this study it has been shown that these significant variations are genetically controlled. So if desired the industry could take this into further consideration and development in breeding for optimal fiber properties. Although today, this is not common.

5. Conclusion

The results from this study suggest that it is not the same gene that control apical and cambial growth rhythm of Norway spruce. Thus earlier bud flushing did not mean significantly earlier cambial activity than later bud flushing.

From the process of studying different techniques for sample preparation in the laboratory, the razorblade cutting method can be recommended. It was definitely timesaving and sufficiently accurate.

Following-up projects

A large job with collecting the material is done, and a foundation is made for further investigations over a longer period of time. Some of the material sampled was not investigated because the timeframe did not allow more measurements. So, there are opportunities to get further on with for example testing the impact of sampling from different compass directions.

The fact that one of the clones did produce both numerous and narrow tracheids would also be interesting to take into further investigation. The results can be used as an indicator for later surveys.

6. References

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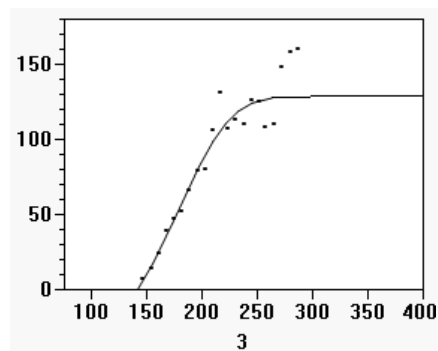
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Appendix 1:

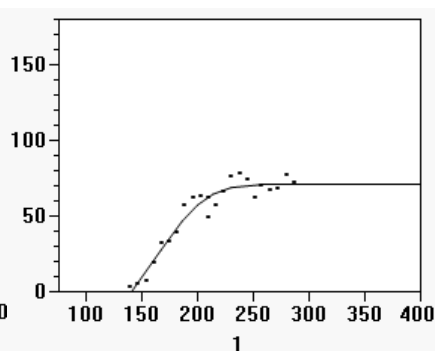
Budflush	Clone	Tree ID	Initiation	Initiation Latewood 1	Initiation Latewood 2	Latewood 1 %	Latewood 2 %	Cessation date	Mean total cells	Ringwidth µm
Very early	337	4	19 May	4 Aug.	20 July	5 %	10 %	13 Sept.	103,2	3032
	337	9	19 May	9 July	5 July	9 %	17 %	8 Sept.	84,7	1760
	337	11	26 May	4 July	30 June	7 %	13 %	8 Sept.	56,4	2368
	337	15	19 May	11 Sept.	30 Aug.	3 %	6 %	8 Sept.	137	3755
Mean			19 May	29 July	22 July	5 %	10 %	28 Aug.	95,5	2729
Early	320	1	19 May	27 July	16 July	5 %	9 %	8 Sept.	71	4463
	320	5	19 May	21 July	9 July	7 %	18 %	8 Sept.	65,5	1548
	320	12	19 May	27 July	16 July	5 %	11 %	25 Aug.	73,5	2713
	320	16	12 May	3 July	24 July	8 %	14 %	1 Sept.	101,8	2277
Mean			12 May	27 July	17 July	6 %	12 %	30 Aug.	77,9	2750
Late	323	2	12 May	25 July	18 July	7 %	18 %	8 Sept.	75,8	1548
	323	6	19 May	29 July	16 July	4 %	7 %	8 Sept.	62,75	2902
	323	10	12 May	15 Aug.	31 July	6 %	11 %	13 Sept.	94,1	2177
	323	13	26 May	8 Aug.	19 July	7 %	12 %	1 Sept.	63,2	2329
Mean			12 May	8 Aug.	26. July	6 %	11 %	7 Sept.	74	2239
Very Late	127	3	26 May	11 Aug.	2 Aug.	6 %	8 %	13 Sept.	128,7	2195
	127	7	19 May	23 July	17 July	12 %	21 %	22 Sept.	111,8	1905
	127	8	19 May	27 Aug.	12 Aug.	7 %	18 %	13 Sept.	114,2	1548
	127	14	19 May	24 July	19 July	4 %	8 %	13 Sept.	115,4	2646
Mean			19 May	2 Aug.	25 July	7 %	13 %	27 Aug.	117,5	2073

Appendix 2:

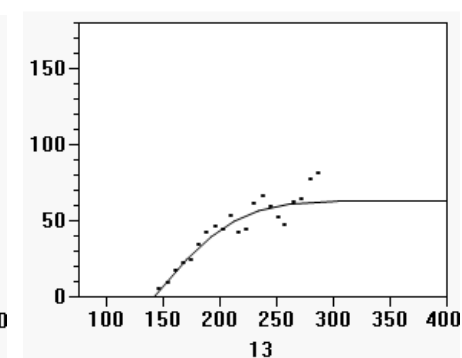
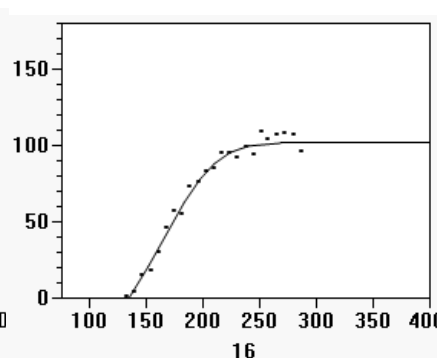
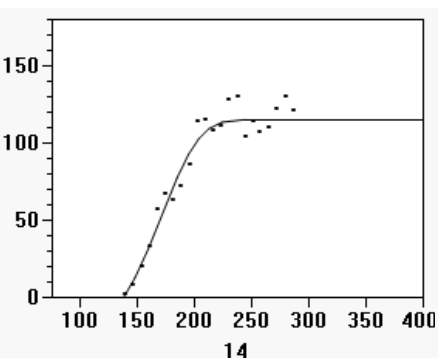
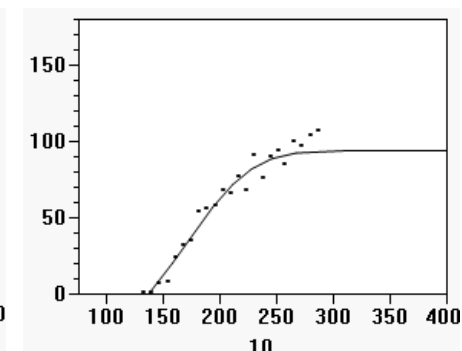
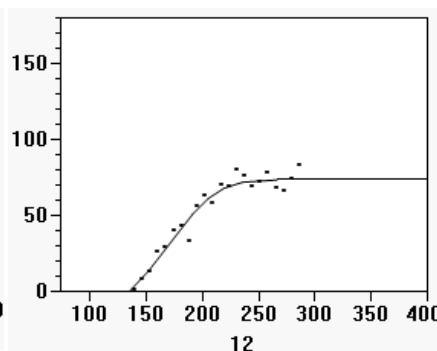
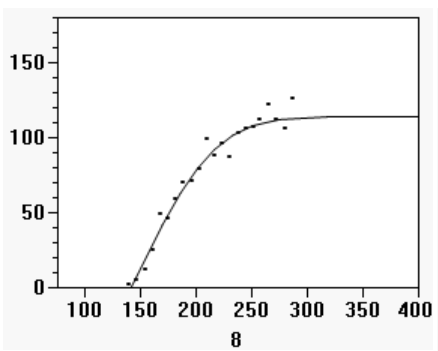
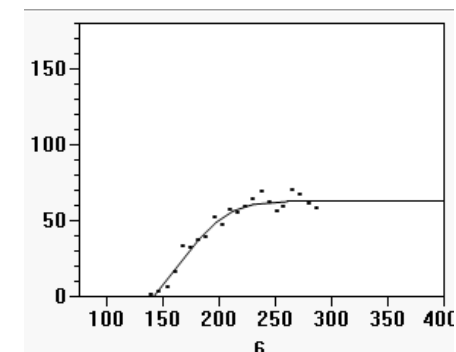
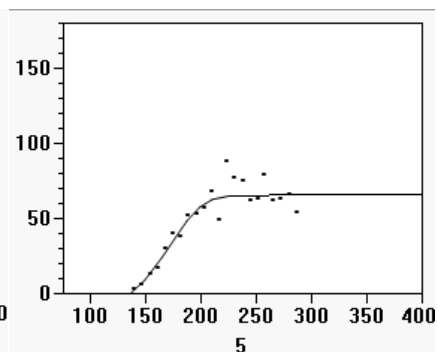
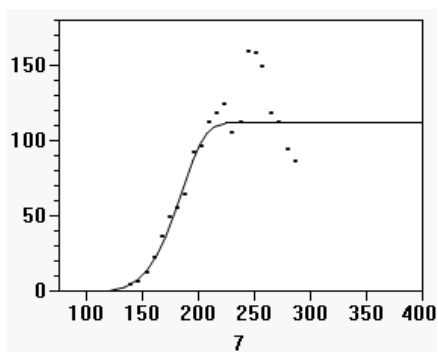
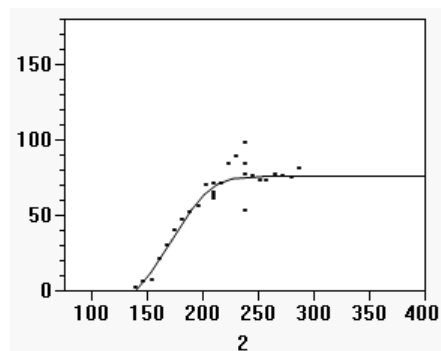
Clone 127:



Clone 320:

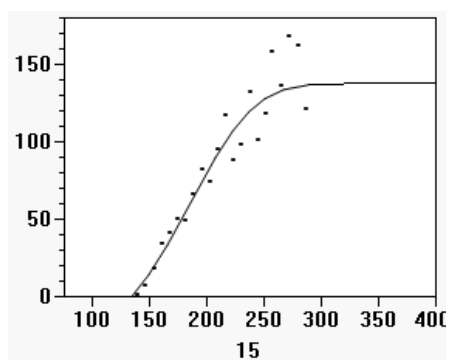
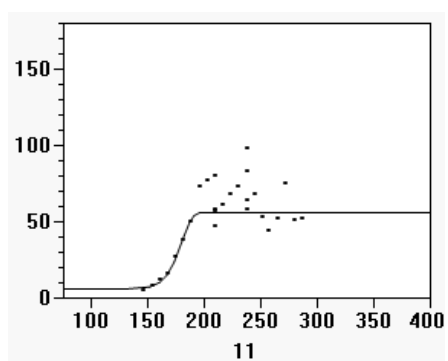
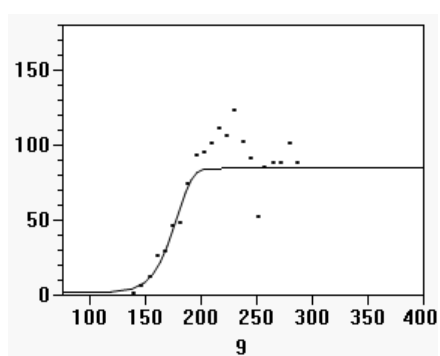
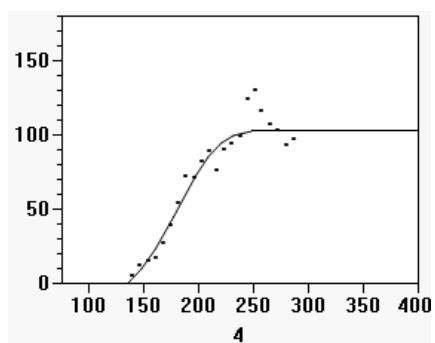


Clone 323:



Appendix 3:

Clone 337:



Appendix 4:

An overview of the different sampling dates with corresponding daynumber used in the analysis, and weeknumber.

Daynumber	Date	Week
12	12 May	19
19	19 May	20
26	26 May	21
33	2 June	22
40	9 June	23
47	16 June	24
54	23 June	25
61	30 June	26
68	7 July	27
75	14 July	28
82	21 July	29
89	28 July	30
96	4 August	31
103	11 August	32
110	18 August	33
117	25 August	34
124	1 September	35
131	8 September	36
137	14 September	37
145	22 September	38
152	29 September	39
159	6 October	40
166	13 October	41